Current Biology 20, 1740–1744, October 12, 2010 ©2010 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2010.08.036

Report

Individual-Level Bet Hedging in the Bacterium *Sinorhizobium meliloti*

William C. Ratcliff[1,*] and R. Ford Denison[1]

[1]Ecology, Evolution and Behavior, University of Minnesota, Minneapolis, MN 55108, USA

Summary

The expression of phenotypic variability can enhance geometric mean fitness and act as a bet-hedging strategy in unpredictable environments [1]. Metazoan bet hedging usually involves phenotypic diversification among an individual's offspring [2–6], such as differences in seed dormancy. Virtually all known microbial bet-hedging strategies, in contrast, rely on low-probability stochastic switching of a heritable phenotype by individual cells in a clonal group [7–10]. This is less effective at generating within-group diversity when group size is small. Here we describe a novel microbial bet-hedging behavior that resembles individual-level metazoan bet hedging. *Sinorhizobium meliloti* stores carbon and energy in poly-3-hydroxybutyrate (PHB) as a contingency against carbon scarcity [11]. We show that, when starved, dividing *S. meliloti* bet hedge by forming two daughter cells with different phenotypes. These have high and low PHB levels and are suited to long- and short-term starvation, respectively. The low-PHB cells have greater competitiveness for resources, whereas the high-PHB cells can survive for over a year without food, perhaps until a legume host is next available.

Results

The bacterium *Sinorhizobium meliloti* fixes nitrogen symbiotically within legume root nodules. Between legume hosts, *S. meliloti* lives saprophytically in the soil, where a lack of reduced carbon can limit reproduction [12]. As a contingency against carbon limitation, *S. meliloti* accumulates large amounts (>50% cell dry weight) of the storage compound poly-3-hydroxybutyrate (PHB; Figure 1A), which can support reproduction and survival during starvation [11]. Rhizobia escaping senescing nodules may quickly find a new host to nodulate or may first have to persist in the soil, perhaps for a year or more, until a suitable host is encountered. The optimal use of PHB depends on the duration of C limitation: short-term starvation favors immediate use of PHB for reproduction, increasing competiveness for exogenous resources and nodulation, whereas long-term starvation instead favors conservation of PHB for survival. When the duration of starvation fluctuates unpredictably (because of either fluctuation in saprophytic resources or nodulation opportunities), the high variation in fitness that results from exclusive reliance on either PHB-use strategy will result in low long-term geometric mean fitness. A bet-hedging strategy in which some of a cell's offspring forgo reproduction and conserve PHB for survival could be adaptive, increasing geometric mean fitness by reducing variation in fitness over generations [1, 2].

*Correspondence: ratcl009@umn.edu

Asymmetric Division in Response to Starvation

When starved, initially high-PHB *S. meliloti* (Figure 1A) differentiated into discrete high- and low-PHB phenotypes (Figure 1B). Some PHB was used in the process, so PHB levels in the high-PHB subpopulation were lower than those in the initial population (Figures 1A and 1B). Reproduction mainly produced new low-PHB cells, which increased to 195% of the original cell count. The number of high-PHB cells, which contained 16 times as much PHB as the low-PHB cells (Figure 1B; $t = 34.7$, $p < 0.0001$, $n = 6$, t test), remained constant during this period, at 96% of the original number of starved rhizobia. Additional experiments using flow cytometry and fluorescence microscopy confirmed the bimodal distribution of PHB levels in the population and showed that both populations consisted of intact cells, based on their ability to exclude propidium iodide (see Figures S1A–S1F available online). The rhizobia remained differentiated into discrete high- and low-PHB phenotypes after more than 500 days of starvation, demonstrating that this phenotypic dimorphism is stable.

The observed bimodal distribution of PHB per cell could be generated either by individual high-PHB rhizobia dividing asymmetrically to produce both high- and low-PHB offspring (individual-level diversification) or by a stochastic process in which some rhizobia consume all PHB for reproduction, whereas some of their clonemates refrain from reproducing (population-level diversification). To differentiate between these two hypotheses, we followed the fate of immobilized high-PHB rhizobia reproducing in starvation buffer over a 24 hr period by microscopy. PHB was preferentially retained in the old-pole cells (Figures 1C–1E), with the median PHB content of new-pole cells only 40% of that of their old-pole parental cells ($t = 7.09$, $p < 0.0001$, $n = 33$, matched-pairs t test). We therefore interpret the high-PHB subpopulation (Figure 1B) with 96% of the original numbers as old-pole cells and the low-PHB subpopulation with 195% of the original numbers as their daughters and granddaughters.

Fitness of Each Phenotype during Long and Short Starvation

To determine whether this asymmetric allocation of PHB among daughter cells can serve as a mechanism for bet hedging (defined and discussed below) during starvation of variable duration, we examined the survival of each phenotype during long- versus short-term starvation. Rhizobia were starved for either 14 or 528 days, and then viability was assessed microscopically with YO-PRO-1, a fluorescent green viability stain. After 14 days, there was no detectable difference in the viability of high- and low-PHB cells (Figure 2A; $t = 0.96$, $p = 0.37$, $n = 10$, t test). After 528 days, high-PHB cells still constituted 30% of the population. These high-PHB cells had a 5-fold survival advantage (Figure 2A; $t = 9.5$, $p < 0.0001$, $n = 10$, t test). In fact, viability of cells in the high-PHB fraction was still 79% of what it had been on day 14. The phenotype that maintains high PHB levels appears to be well suited to long-term starvation.

The low-PHB phenotype can't survive long-term starvation, but are there conditions under which it outperforms the

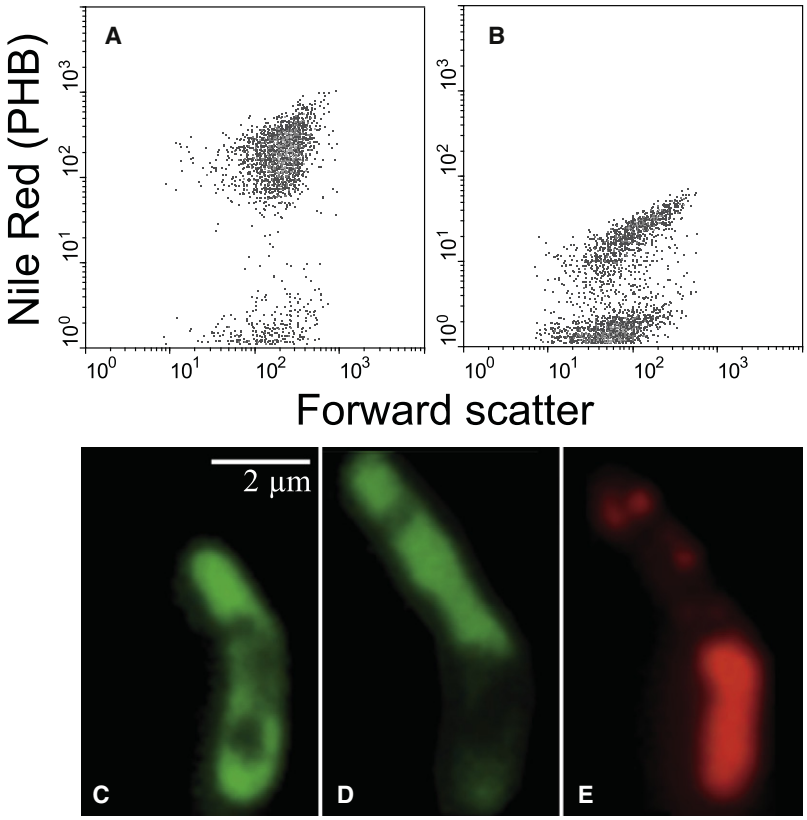

Figure 1. *Sinorhizobium meliloti* Differentiates into High- and Low-Poly-3-Hydroxybutyrate Phenotypes when Starved

PHB was measured flow cytometrically using the fluorescent probe Nile Red (NR).

(A) All cells initially contained large amounts of poly-3-hydroxybutyrate (PHB).

(B) After 29 days of starvation, these cells were differentiated into distinct high- or low-PHB phenotypes.

(C–E) GFP-labeled *S. meliloti* from a uniformly high-PHB population was immobilized, starved, and imaged after 0 hr (C) and 24 hr (D and E).

(E) NR staining indicates that PHB allocation is asymmetric: the new-pole cell contains little PHB, whereas the old-pole cell retains the majority of the remaining maternal PHB.

high-PHB phenotype? To answer this question, we used two independent methods to generate populations of *S. meliloti* that varied in the percentage of cells with the high-PHB phenotype and then subcultured each population into rich media, along with a common competitor. In the first method, GFP-labeled *S. meliloti* 1021 rhizobia were starved at high (5×10^6 cells/ml) or low (10^5 cells/ml) density for 3 days, resulting in populations containing 61% and 34% high-PHB cells, respectively. After 24 hr of growth, we determined their fitness relative to a common competitor (unlabeled *S. meliloti* 1021). The GFP-labeled subpopulation with a smaller percentage of high-PHB cells was significantly more fit than the population with a majority of high-PHB cells (Figure 2B; $t = 16.7$, $p < 0.0001$, $n = 9$, t test). Next we used density gradient centrifugation to fractionate three replicate populations of starved *S. meliloti* into subpopulations either enriched or depleted in high-PHB rhizobia. Again, fractions with a majority of low-PHB cells were significantly more fit (e.g., Figure 2C; $p < 0.0001$, $t = 11.13$, $n = 10$, t test; all three replicates significant with $p < 0.05$ after Bonferonni correction).

There are two potential causes for the reduced competitiveness of high-PHB rhizobia during growth. First, they could be less physiologically suited for immediate growth and thus take longer to respond to resources. Second, they may respond to resources as quickly as low-PHB rhizobia but then reproduce more slowly, exhibiting a reduced exponential rate of increase. We examined both factors by generating 27 populations of starved *S. meliloti* 1021 that varied in the frequency of high-PHB rhizobia, and then we subcultured them into rich media. Over the next 30 hr, we measured the rate of population growth and determined the duration of the lag phase and the exponential rate of increase for each population. Populations containing a larger fraction of high-PHB cells had a significantly longer lag phase, delaying growth by an additional 0.96 min for each additional percentage of high-PHB cells in the population (Figure S2B; $p = 0.044$, $n = 27$, linear regression). Extrapolated to 0% and 100% high-PHB cells, this suggests a 96 min difference in lag phase between the two phenotypes. Cellular phenotype, however, had no detectable effect on their exponential rate of increase after growth began ($p = 0.16$, $n = 27$, linear regression). It appears that the low-PHB phenotype is primed for rapid reproduction as soon as resources are encountered.

Ability of Both Phenotypes to Rediversify

If each phenotype was completely heritable, then diversification would be the result of an evolutionary radiation, not bet hedging [1]. We thus determined whether high- and low-PHB phenotypes are capable of rediversifying when put through an additional round of growth and starvation. Using density-gradient centrifugation, we generated fractions that were either enriched (48.4%) or depleted (11.2%) in high-PHB cells from a single starved, dimorphic population of *S. meliloti*. These fractions were plated on rich media (TY), and 48 single-cell isolates were randomly selected from each fraction. All isolates accumulated PHB when grown in M9 mannitol media and then diversified into high- and low-PHB cells. Further, the phenotype of the founding cell did not affect this diversification: isolates drawn from the enriched and depleted fractions formed populations in which, after 29 days of starvation, the high-PHB phenotype constituted an average of 88.3% and 83.3% of the original cell count, respectively ($t = 0.68$, $p = 0.50$, $n = 95$, t test), and the low-PHB phenotype constituted 146% and 154.3% of the original cell count, respectively ($t = 0.44$, $p = 0.66$, $n = 95$, t test; Figure S2B). The high- and low-PHB phenotypes do not show even epigenetic inheritance.

Discussion

Starving *Sinorhizobium meliloti* cells face a tradeoff between immediate reproduction and long-term survival. This tradeoff is driven by two distinct mechanisms: first, a given amount of PHB may be either catabolized to power reproduction or conserved to enhance long-term survival. A mismatch

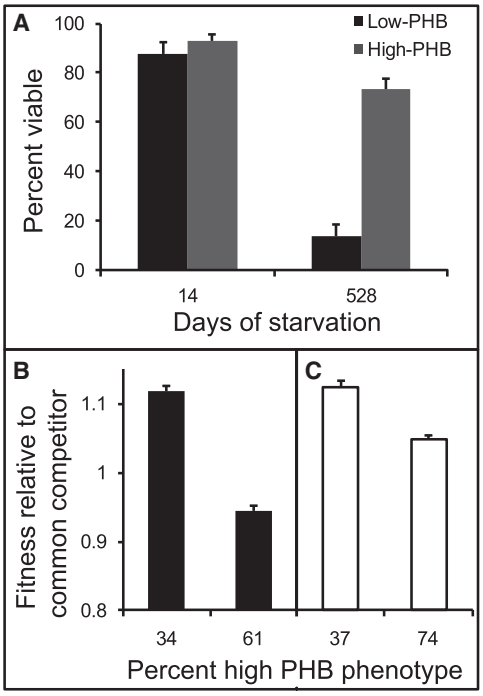

Figure 2. High- and Low-PHB Phenotypes Are Suited to Long- and Short-Term Starvation, Respectively

(A) The high-PHB phenotype survives long-term starvation with little mortality. Shortly after phenotypic differentiation (14 days of starvation), both high- and low-PHB rhizobia were still equally viable, as shown by viability staining. However, after 528 days of starvation, significantly more high-PHB cells were viable than low-PHB cells.

(B and C) However, the high-PHB phenotype is less competitive for exogenous resources when starvation is short term (3 days; B and C). Populations of starved, GFP-labeled *S. meliloti* that varied in the frequency of high-PHB cells were subcultured into rich media along with an unlabeled but otherwise isogenic common competitor. Shown is the relative fitness (ratio of Malthusian parameters) of the test population of a common competitor after 24 hr growth. Variation in the frequency of high-PHB cells was generated in two independent experiments by starvation at different cell densities (B) or fractionation of a single population by buoyant density (C). Two additional replicates from (C) gave qualitatively similar results (not shown). Plotted are means ± SEM.

between PHB use and conditions can be costly. Using all stored PHB for reproduction can result in death when starvation is long term. When starvation is only short term, however, cells that use PHB for reproduction will have more descendants. Second, we found that metabolically active bacteria are more competitive for exogenous resources but are more susceptible to exhausting their energy reserves and dying of starvation. Our data show that, when starved, individual *S. meliloti* divide asymmetrically, producing a low-PHB offspring (which may quickly divide again) and an old-pole high-PHB cell. Low-PHB offspring are primed for further saprophytic reproduction, which could increase opportunities for legume nodulation, whereas the less-active old-pole high-PHB cell is capable of surviving for over a year without food. The apparent dormancy of the old-pole cell would enhance survival under starvation even if initial resource allocation was equal, but survival is further enhanced by greater allocation of PHB to this cell.

The term "bet hedging" is sometimes used loosely, but a rigorous definition includes lower expected arithmetic mean fitness, as well as greater expected geometric mean fitness [1, 2]. If the duration of starvation is unpredictable, then asymmetric division into both high- and low-PHB phenotypes (with associated physiological differences favoring survival versus reproduction) could serve as a bet-hedging mechanism by reducing variance in maternal inclusive fitness across starvation events. Without a record of the long-term frequency and severity of starvation faced by *S. meliloti* in the field, however, we cannot determine conclusively whether this behavior evolved as a result of selection for diversification bet hedging. This limitation is nearly ubiquitous in studies of bet hedging: to our knowledge, only two studies [2, 13] have shown that a putative bet-hedging trait decreases a genotype's arithmetic mean fitness while increasing geometric mean fitness in the environment in which the trait evolved. Nonetheless, the asymmetric division in starving *S. meliloti* and optimization of low- and high-PHB phenotypes for growth and stress resistance, respectively, strongly suggest that this behavior evolved as a risk-spreading adaptation.

This is the first example of a microbial bet-hedging mechanism that allows a single cell to effectively express a diversification strategy (via division into daughter cells with contrasting phenotypes) when exposed to stress. Most putative microbial bet-hedging strategies result from phase variation [7], contingency loci [8], and epigenetically inherited phenotypic bistability [9, 10]. With these mechanisms, diversification is driven by low-probability (typically 10^{-1} to 10^{-5} per individual per generation) stochastic switching that generates phenotypic diversity among a group of genetically identical cells rather than by phenotypic plasticity that responds adaptively to current conditions. Except at the higher frequency end of this range (10^{-1}), a small group founded by a single phenotype is unlikely to contain even a single variant, making the entire clonal group subject to extinction. Further, the ability of stochastic switching to create phenotypic diversity depends on adequate generational turnover between selective events [14]. With few generations between events that eliminate one or the other phenotype, there is little opportunity for the production of offspring with contrasting phenotypes and thus minimal diversification.

In contrast, the classic diversification strategies commonly found in metazoans [2–6] generate diversity among progeny every generation and thus are effective even when group size is small and stress frequent. For example, a plant producing as few as two seeds might still have significant variation in dormancy among its offspring. For rhizobia, dispersal in the rhizosphere and subsequent competition for different nodulation opportunities would favor similar bet hedging, especially during starvation, when saprophytic reproduction (and hence the number of generations during which stochastic switching can generate diversity) in a patch of the rhizosphere is limited. Successful nodulation requires that rhizobia encounter the root of a compatible legume during a period of active nodulation and outcompete other rhizobia for root entry [15]. Individual rhizobial cells released from nodules early in a growing season may, by forming both high- and low-PHB phenotypes when starved, take advantage of late-season nodulation opportunities the same year while also increasing the chances of surviving until new hosts are next available. In agricultural environments, viable rhizobial populations can persist in soil for years without a compatible host [16]. To some extent, this may be due to prolonged survival of individual cells.

Experimental Procedures

Rhizobial Strains and Culture

All work was done with *Sinorhizobium meliloti* strain 1021 and the GFP-labeled derivative pDG71 [17]. Before starvation, rhizobia were cultured in 50 ml of M9 minimal media [18] + 10 g/l mannitol in 125 ml Erlenmeyer flasks shaken at 100 rpm at 22°C for 6 days. pDG71 was cultured with the presence of 5 μg/ml tetracycline.

Starvation

S. meliloti 1021 cells were harvested by centrifugation from growth flasks and double washed in carbon-free M9 media (containing no mannitol, 0.1 g/l thiamine, and high-pressure liquid chromatography-grade water). Caution was taken to ensure that starvation media remained carbon free. All glassware used was acid washed (0.6 M HCl for 1 hr), rinsed in deionized (DI) water, and pyrolyzed at 550°C overnight. All starvation experiments were initiated at a density of 5×10^5 to 5×10^6 cells/ml unless otherwise noted, with cell density determined by either plate counting or fluorescence-activated cell sorting analysis, as previously described [11].

Flow Cytometry

All flow cytometry was carried out on a Becton Dickinson FACSCalibur. PHB per cell was quantified by staining with the red fluorescent dye Nile Red (NR) as previously described [11]. Viability analysis was conducted by using the red fluorescent DNA stain propidium iodide (PI) [11]. 1% of the PI stock solution (1 mg PI/ml in H_20) was added to cells at 5×10^5 to 5×10^6 cells/ml, incubated for 5–10 min, and then assayed flow cytometrically, collecting data on the FL3 detector (>670 nm filter). Live and fixed (30% EtOH for 30 min) controls were run to determine the fluorescence range of viable and killed cells, and viability for all samples was determined by gating.

Following the Fate of PHB in Pairs of Dividing Cells

To determine whether high- and low-PHB phenotypes were generated by individual-level or stochastic processes, we developed a method to follow individual cells during culture in liquid media. We employed 8-well Nunc Lab-Tek II chamber slides, which allow culture of rhizobia on a coverslip under 0.5 ml liquid media. The inner surface of each slide was first marked with an ~0.3 mm diameter dot from a permanent marker (Sharpie), allowing us to repeatedly find the same field of view, and then treated with poly-D-lysine (0.1 mg/ml for 5 min, removed via pipette and double washed in sterile DI water). Slides were air dried in a laminar flow hood, and 10 μl of high-PHB rhizobia at 10^8 cells/ml was placed over the marked region of the slide. Rhizobia were allowed to adhere to the poly-D-lysine-treated coverslip for 30 min, and then the medium was removed by pipette and the marked region was rinsed twice with 50 μl of C-free M9 to remove free cells. Rhizobia were starved in 300 μl C-free M9. Rhizobia were imaged on an Olympus IX70 inverted epifluorescence microscope equipped with a 4MP SPOT digital camera at time 0 and after 24 hr. We quantified PHB/cell by staining with Nile Red—first fixing with 135 μl 95% EtOH (resulting in a 30% EtOH solution) for 30 min, then adding 1% NR stock solution (1 mg/ml in dimethyl sulfoxide [DMSO]), then incubating for 60 min, and then reimaging. PHB/cell in the old and new cell pairs was quantified by measuring the median NR fluorescence intensity of each cell in ImageJ after thresholding to remove background fluorescence.

Viability during Long and Short Starvation

S. meliloti 1021 cells starved in M9-C were assayed for PHB content and viability simultaneously via microscopy, using the red fluorescent PHB stain Nile Red and green fluorescent viability stain YO-PRO-1 (shown to be comparable in action to propidium iodide, Figure S2C). 1% of the staining solution (1 mg/ml Nile Red and 0.1 mM YO-PRO-1 in DMSO) was added to rhizobia at $\sim 5 \times 10^6$ cells/ml and incubated for 60 min at room temperature, and then excess stain was removed by centrifugation. For each sample, at least 30 fields of view were randomly imaged on an Olympus IX70 microscope for both NR and YO-PRO-1 fluorescence. RGB-stacked images were decomposed into their three single color elements, and only the red and green components were kept for NR and YO-PRO-1 fluorescence emissions, respectively. These two images were combined into a single stack and registered in ImageJ. Both NR and YO-PRO-1 emit a small amount of fluorescence in the green and red spectra, respectively; we removed this potentially confounding effect with the Spectral Unmixing plug-in version 1.2 (written by J. Walter) in ImageJ. Median NR and YO-PRO-1 fluorescence intensity were measured for each cell, and, as with flow cytometry, viability and high or low PHB content were determined by gating.

Rhizobial Fitness during the Starvation-Growth Transition

Three populations of starved pDG71 with bimodal PHB content were separated into high (1.138–1.119 g/ml) and low (≤1.109 g/ml) buoyant density fractions, and thus populations enriched and depleted in high-PHB cells, using density gradient centrifugation as described in [11]. For each fraction, five replicate competitions were established. Of pDG71 cells, 10^5 was co-inoculated into 1 ml of tryptone yeast (TY) medium (per liter: 5 g tryptone, 3 g yeast extract, 0.66 g $CaCl_2 \times 2\ H_2O$) with an equal number of *S. meliloti* 1021, previously starved for 3 days at 5×10^6 cells/ml. After 24 hr incubation at 30°C, the population size of both strains was determined by flow cytometry. pDG71 cells were counted by gating on FL1, whereas *S. meliloti* 1021 cells were counted by subtracting GFP-labeled rhizobia from a count of all rhizobia (determined by a forward scatter × side scatter (FSC × SSC) gate). Relative fitness of each pDG71 population to the common competitor was calculated by taking the ratio of the Malthusian parameters of growth [19]. In a separate experiment, we generated variation in the frequency of the high-PHB phenotype by starving pDG71 at high (5×10^6) or low (10^5) density for 3 days. pDG71 starved at high density reproduced less and formed populations containing a larger fraction of high-PHB cells. Competitions and analyses were conducted in the same manner.

Lag Phase and Exponential Growth Rate of Both Phenotypes

Variation in the percentage of high-PHB cells was generated by starving *S. meliloti* 1021 at high (5×10^6), medium (5×10^5), and low (10^5) densities for 3 days. Nine replicates were starved at each density, resulting in 27 populations that varied in the fraction of high-PHB cells. These were subcultured into rich media (TY) and incubated at 30°C for 30 hr. Each population was sampled at 0, 4, 12, 18, 24, and 30 hr of starvation, and population size was determined via flow cytometry. Cell doublings for 12–30 hr growth were compared to the average of 0 and 4 hr, in which no growth was detected. Gompertz growth curves, a three-parameter model commonly used to describe bacterial growth dynamics [20], were fit to the number of cell doublings over time using the iterative parameter-fitting algorithm in JMP 7.0 (Statistical Analysis Software Institute). The parameters of the Gompertz growth curve, which describes the duration of lag phase and the rate of exponential growth, were used as the response measurements for the experiment.

Statistical Analyses

All statistics were computed in JMP 7.0. All t tests were two tailed.

Supplemental Information

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.cub.2010.08.036.

Acknowledgments

The authors thank Michael Travisano and Tony Dean for helpful discussions, Daniel Gage for providing GFP-labeled *Sinorhizobium meliloti* 1021 pDG71, Mitch Hoverman for laboratory assistance, and the University of Minnesota Imaging Center. This work was supported by National Science Foundation (NSF) grants DEB-0918897 and DEB-0808234 and an NSF predoctoral fellowship (W.C.R.).

Received: April 13, 2010
Revised: July 30, 2010
Accepted: August 17, 2010
Published online: September 23, 2010

References

1. Seger, J., and Brockmann, H.J. (1987). What is bet-hedging? In Oxford Surveys in Evolutionary Biology, P. Harvey and L. Partridge, eds. (New York: Oxford University Press), pp. 182–211.
2. Simons, A.M. (2009). Fluctuating natural selection accounts for the evolution of diversification bet hedging. Proc. Biol. Sci. *276*, 1987–1992.
3. Hopper, K.R. (1999). Risk-spreading and bet-hedging in insect population biology. Annu. Rev. Entomol. *44*, 535–560.
4. Lips, K. (2001). Reproductive trade-offs and bet-hedging in Hyla calypsa, a Neotropical treefrog. Oecologia *128*, 509–518.

5. Crean, A.J., and Marshall, D.J. (2009). Coping with environmental uncertainty: Dynamic bet hedging as a maternal effect. Phil. Trans. Roy. Soc. B. Biol. Sci. *364*, 1087–1096.
6. Laaksonen, T. (2004). Hatching asynchrony as a bet-hedging strategy: An offspring diversity hypothesis. Oikos *104*, 616–620.
7. van der Woude, M.W., and Bäumler, A.J. (2004). Phase and antigenic variation in bacteria. Clin. Microbiol. Rev. *17*, 581–611.
8. Moxon, R., Bayliss, C., and Hood, D. (2006). Bacterial contingency loci: The role of simple sequence DNA repeats in bacterial adaptation. Annu. Rev. Genet. *40*, 307–333.
9. Gordon, A.J.E., Halliday, J.A., Blankschien, M.D., Burns, P.A., Yatagai, F., and Herman, C. (2009). Transcriptional infidelity promotes heritable phenotypic change in a bistable gene network. PLoS Biol. *7*, e1000044.
10. Veening, J.W., Stewart, E.J., Berngruber, T.W., Taddei, F., Kuipers, O.P., and Hamoen, L.W. (2008). Bet-hedging and epigenetic inheritance in bacterial cell development. Proc. Natl. Acad. Sci. USA *105*, 4393–4398.
11. Ratcliff, W.C., Kadam, S.V., and Denison, R.F. (2008). Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. FEMS Microbiol. Ecol. *65*, 391–399.
12. Veen, A.H., and Lindenmayer, A. (1977). Diffusion mechanism for phyllotaxis: Theoretical physico-chemical and computer study. Plant Physiol. *60*, 127–139.
13. Beaumont, H.J.E., Gallie, J., Kost, C., Ferguson, G.C., and Rainey, P.B. (2009). Experimental evolution of bet hedging. Nature *462*, 90–93.
14. Saunders, N.J., Moxon, E.R., and Gravenor, M.B. (2003). Mutation rates: Estimating phase variation rates when fitness differences are present and their impact on population structure. Microbiology *149*, 485–495.
15. Gage, D.J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol. Mol. Biol. Rev. *68*, 280–300.
16. Kucey, R.M.N., and Hynes, M.F. (1989). Populations of *Rhizobium leguminosarum* biovars *phaeoli* and *viceae* in fields after bean or pea in rotation with nonlegumes. Can. J. Microbiol. *35*, 661–667.
17. Gage, D.J. (2002). Analysis of infection thread development using Gfp- and DsRed-expressing *Sinorhizobium meliloti*. J. Bacteriol. *184*, 7042–7046.
18. Miller, J.H. (1992). A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria (New York: Cold Spring Harbor Laboratory Press).
19. Lenski, R.E., Rose, M.R., Simpson, S.C., and Tadler, S.C. (1991). Long-term experimental evolution in Escherichia coli. I. Adaptation and divergence during 2,000 generations. Am. Nat. *138*, 1315–1341.
20. Zwietering, M.H., Jongenburger, I., Rombouts, F.M., and van 't Riet, K. (1990). Modeling of the bacterial growth curve. Appl. Environ. Microbiol. *56*, 1875–1881.